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(54) Title: PROMOTION OF WOUND HEALING WITH HUMAN EPIDERMAL GROWTH FACTOR PREPARED FROM RECOMBINANT DNA

(57) Abstract

Treatment of wounds which result from cuts, abrasions, burns, skin ulcers, skin grafts, and the like, and the promotion of healing. One of the technical problems experienced in this area has been the long healing times generally encountered. Some sensitive areas, such as the cornea, are difficult to treat over prolonged periods. This invention provides a solution to this problem by teaching a composition and use of the composition to promote rapid healing of skin, stromal and corneal wounds, by promoting rapid regeneration of the affected tissue. The method involves topical application of a composition which contains a particular polypeptide. The polypeptide is produced using recombinant DNA techniques, specifically through use of a plasmid designated pY EGF-23. The polypeptide exhibits mitogenic activity similar to that of natural epidermal growth factor. The method and composition have been effectively used in treating both skin wounds and corneal wounds.

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PROMOTION OF WOUND HEALING WITH HUMAN EPIDERMAL GROWTH FACTOR PREPARED FROM RECOMBINANT DNA

Background of the Invention

1. Field of the Invention

Wounds resulting from cuts, abrasions, burns, skin ulcers, skin grafts, and the like, can affect large areas of the skin and require lengthy periods to heal. Long healing times are a particular problem with wounds on sensitive areas, such as corneal wounds, which are difficult to treat over prolonged periods. For these reasons, there has been a long felt need for a pharmacological agent which will promote rapid healing of skin and corneal wounds.

Heretofore, a number of research groups have investigated the use of epidermal growth factor obtained from mouse salivary glands (mEGF) to promote the regeneration of epidermal wounds. Thus far, the treatment of relatively large wounds resulting from abrasions, burns, and the like with mEGF has proved to be of only marginal value in promoting wound healing. Although the use of mEGF in promoting healing of corneal epithelial wounds has been more promising, such treatment has not been shown to be effective in accelerating the rate of healing of corneal stromal wounds.

It would thus be desirable to provide a method and agent for treating epithelial, stromal and corneal wounds which will promote the rapid healing of these wounds. In particular, it would be desirable to provide such an agent in large quantities and in formulations which are suitable for treatment of the affected area.

2. Description of the Prior Art

A comparison of human and mouse epidermal growth factor (EGF) and a discussion of their activities in vivo and in vitro are presented in Hollenberg, "Epidermal Growth Factor-Urogastrone, A Polypeptide Acquiring Hormonal Status" eds., Academic Press, Inc., New York (1979) pp. 69-110. See also, Carpenter, "Epidermal Growth Factor" in: Handbook of Experimental Pharmacology, Vol. 57, Baserga, ed., Springer Verlag, Berlin (1981) pp. 90-132; and Carpenter (1979) Ann. Rev. Biochem. 48:193-216.

Various studies have examined the use of mEGF in treating epidermal wounds. Greaves (1980) Clin. Exp. Dermatol. 5:101-103 applied mouse EGF on blister wounds on human subjects. The mEGF in saline solution was applied once daily until the wounds healed. No acceleration in the growth of the epidermal layer was observed. Topical application of mouse EGF to open wounds in mice has been found to promote healing in various degrees. See, e.g., Niall et al. (1982) J. Surg. Res. 33:164-169; Thornton et al. (1981) Burns 8:156-160.

The use of mouse EGF to promote the healing of corneal epithelial wounds has been described. See, e.g., Daniele et al. (1979) Graefes Archives Ophthalmologie 210:159-165; Ho et al. (1974) Invest. Ophthalmol. 13:804-809; Elliott (1980) Trans. Amer. Ophthamol. Soc. 30:629-656; and Gospodarowicz et al. (1977) Exp. Eye Res. 25:75-89.

Summary of the Invention

Methods and compositions for treating epithelial and stromal wounds to promote their rapid healing are provided. The method utilizes a treatment composition including a purified polypeptide having mitogenic activity capable of promoting the growth of both the epidermal and dermal layers of the skin as well as the epithelial and stromal layers of the

cornea. Healing occurs, in part, as a result of the differentiation of epithelial and stromal cells into fibroblasts to cause the formation of scar tissue. The polypeptide is produced by recombinant DNA techniques, typically utilizing a synthetic gene having a nucleotide sequence based on the known amino acid sequence of human epidermal growth factor. The compositions are applied topically to the affected area and include suitable carriers or bases. For general treatment of areas other than the cornea, the carrier will usually be an ointment or a cream, typically including an antibacterial agent. For corneal treatment, the carrier will be a suitable liquid or ointment.

Description of the Specific Embodiments

The present invention provides a method for treating cutaneous and corneal wounds to accelerate healing of the wounds in humans and other mammals. The method will also find use in treating other epithelial and stromal disruptions, such as chronic ulcers, burns, surgical wounds, and injuries to the hollow, epithelial lined organs, such as the esophogus, stomach, large and small bowels, mouth, and urinary and genital tract. The method relies on the topical application of a treatment composition including a polypeptide having an amino acid sequence and mitogenic activity similar to that of human epidermal growth factor (hEGF). The polypeptide is produced in a microorganism from a gene encoding the amino acid sequence for hEGF. In the exemplary embodiment, the gene is a synthetic gene composed of codons preferentially recognized by yeast, and the microorganism host is yeast. By suitably purifying the resulting polypeptide product, and applying the polypeptide to the affected area in a physiologically-acceptable carrier medium, it has been found that of the rate of the healing process is substantially increased.

Human EGF is a mitogenic polypeptide found in urine which is capable of stimulating the proliferation of keratinocytes and other mammalian epidermal cells in culture. The polypeptide exists as a 53 amino acid form (β-hEGF) and a 52 amino acid form (γ-hEGF), which forms are identical except that γ-hEGF lacks the C-terminal arginine residue found on β-hEGF. The amino acid sequence for both forms is reported in Hollenberg (1979) supra.

As used hereinafter and in the claims, human epidermal growth factor or hEGF will refer to a polypeptide product which is produced in a microorganism and which displays biological activity, e.g., mitogenic activity, similar to natural human epidermal growth factor protein as measured in recognized bioassays. The polypeptide product will have an amino acid sequence which is the same or substantially the same as the natural protein, usually differing by no more than five amino acids, more usually differing by three or fewer amino acids. For the most part, the hEGF amino acid sequence will differ, if at all, by substitutions among the non-polar amino acids, i.e., aliphatic and aromatic amino acids. The deviations from the natural amino acid sequence will not adversely affect the mitogenic activity of the polypeptide product and its ability to promote epithelial healing. Prior to incorporation in a treatment composition, the hEGF polypeptide will be suitably purified (as described below) to remove other proteins and substances recovered from the microorganism host. Purification is essential to assure that undesirable activity resulting from other substances is not present in the treatment composition.

The hEGF polypeptide is obtained by expression of an hEGF gene in a suitable microorganism host, prefereably a yeast host which can provide for secretion of the polypeptide as described below. The

hEGF gene may be chromosomal DNA, cDNA, synthetic DNA, or a combination thereof, e.g., synthetic DNA may be combined with the cDNA to complete the hEGF gene. Conveniently, the present invention will utilize a synthetic DNA sequence encoding for the amino acid sequence of either β-hEGF or γ-hEGF. The hEGF gene will be incorporated in an extrachromosomal element including a replication system recognized by a desired host, typically yeast, and transcriptional and translational regulatory control sequences controlling the expression of the hEGF gene. The extrachromosomal element may include a number of other features, such as selectable markers, which facilitate manipulation of the element. The construction of a number of suitable extrachromosomal elements capable of producing hEGF polypeptides is described in copending application serial no. 522,909, filed on August 12, 1983, assigned to the assignee of the present invention, the relevant portions of which are incorporated herein by reference. Desirably, the extrachromosomal elements of the present invention will include a secretory leader and processing signal sequence in proper reading frame with the hEGF gene in order to provide host translational modification and secretion of the gene product. Secretion facilitates recovery of the polypeptide, allowing isolation from the culture medium without having to lyse the host. Moreover, secretion of the hEGF polypeptide avoids contamination of the EGF with intracellular proteins and other substances which would be released by lysing the microorganism host.

Secretory leader and processing signal sequences suitable for the preferred yeast host will normally be derived from naturally-occurring DNA sequences in yeast which provide for secretion of a polypeptide. Such polypeptides which are naturally secreted by yeast include α-factor, a-factor, acid phosphatase, and the like. If desired, the

naturally-occurring sequence may be modified, for example, by reducing the number of lys-arg pairs which define the processing site (while retaining at least one pair), or by reducing the length of the mRNA leader (while retaining sufficient length to provide for secretion), or by introducing point mutations, deletions or other modifications which facilitate manipulation, e.g., restriction recognition sites. Conveniently, the secretory leader and processing signal sequence may be joined to the hEGF structural gene by providing appropriate cohesive ends on the synthetic structural gene, by means of adaptor molecules, or a combination of both.

After the hEGF polypeptide has been recovered from the microorganism culture, it is necessary to purify the polypeptide to remove foreign proteins and other substances which might have an adverse effect on the compositions of the present invention. As stated above, purification is simplified and enhanced by providing for secretion of the hEGF polypeptide into the culture medium. Purification may then be accomplished as follows.

The yeast culture is centrifuged, and the supernatant medium is concentrated by pressure filtration and ultrafiltration. This concentrated solution, containing the secreted hEGF, is then submitted to ion exchange chromatography and the fractions containing hEGF activity are further purified by high performance liquid chromatography (HPLC). The hEGF peak obtained after HPLC will typically be greater than 95% pure.

The compositions of the present invention will be useful for treating a wide variety of wounds, including substantially all cutaneous wounds, as well as injuries to the cornea and epithelial lined hollow organs. Wounds suitable for treatment result from trauma such as burns, abrasions, cuts, and the like, as

well as from surgical procedures such as surgical incisions and skin grafting. Other conditions suitable for treatment with the present invention include chronic conditions, such as venous stasis ulcers, diabetic ulcers, and other non-healing (trophic) conditions. The compositions will find particular use in treating corneal and scleral wounds, including wounds which affect the epithelial layer, stromal layer, and endothelial layer. Treatment according to the present invention will promote cell division of the endothelial layer of the cornea which was previously thought incapable of growth and regeneration.

The polypeptides will be incorporated in physiologically-acceptable carriers for application to the affected area. The nature of the carriers may vary widely and will depend on the intended area of application. For application to the skin, a cream or an ointment base is usually preferred, suitable bases include lanolin, Silvadene® (Marion) (particularly for the treatment of burns), Aquaphor® (Duke Laboratories, South Norwalk, Connecticut), and the like. If desired, it will be possible to incorporate the hEGF-carrier compositions in bandages and other wound dressing to provide for continuous exposure of the wound to the hEGF. Aerosol applicators may also find use.

For corneal treatment, the carrier will be suitable for application to the eyes. Suitable carriers include ointments, saline solutions, isotonic saline solutions, such as Sorbi-care™ (Allergan Pharmaceuticals) Neodecadron® (Merck, Sharp & Dohme), and the like. A suitable ointment base is sold under the tradename Lacrilube®. The ocular carriers will normally include preservatives, such as benzalkonium chloride and edetate disodium, unless they are formulated immediately prior to application.

Often, it may be desirable to incorporate the hEGF in liposomes to provide for release of the hEGF

over an extended period, typically from 24 to 48 hours. Such incorporation may be particularly desirable when the hEGF is incorporated into a wound dressing as described above. The concentration of polypeptide in the treatment composition is not critical, usually, the polypeptide will be present at from at least 1 µg/ml, usually between 10 µg/ml and 10 mg/ml. The compositions will be applied topically to the affected area, typically as eye drops to the eye or as creams or lotions to the skin. In the case of the eyes, frequent treatment is desirable, usually being applied at intervals of 4 hours or less. On the skin, it is desirable to continually maintain the treatment composition on the affected area during the healing period, with applications of the treatment composition from 2 to 4 times a day, or more frequently.

Optionally, the treatment compositions of the present invention may be combined with effective amounts of anesthetics, antibiotics, antiseptics, and other drugs, typically present at from about 0.001% to 2% by weight.

The following experimental results are offered by way of example, not by way of limitation.

EXPERIMENTAL

Materials and Methods

1. Preparation of hEGF Polypeptide

The hEGF polypeptide utilized in the following experiments was obtained by expression of plasmid pYαEGF-23 in yeast strain AB103. Plasmid pYαEGF-23 was prepared as described in Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646. Plasmid pYαEGF-23 contains a synthetic sequence for mature epidermal growth factor (hEGF) based on the amino acid sequence of EGF reported by Gregory and Preston (1977) Int. J. Peptide Protein Res. 9:107-118.

The hEGF gene is joined in its 5'-end to sequences encoding the promoter and leader region of the yeast mating pheromone α-factor, and in its 3'-end to terminator sequences of the α-factor gene. This expression cassette for hEGF is cloned into a yeast shuttle vector, pC1/1, which contains yeast and bacterial origins of replication and genetic markers (amp^R for bacteria, leu2 for yeast). Yeast cells transformed with plasmid $pY_{\alpha}EGF$-23 efficiently produce and secrete authentic biologically active hEGF into the medium (Brake et al., supra).

2. Purification of hEGF

The human EGF from the yeast cultures was purified by pressure filtration and ultrafiltration, followed by batch adsorption and elution from a carboxylic acid ion-exchange resin (BioRex-70), in 0.1M acetic acid; and high performance liquid chromatography (HPLC). Supernatant broth (180 liters) from the yeast fermentation was submitted to pressure filtration through a cellulose membrane using several Spira/Por unit cartridges of up to 5 sq. ft. (Ultra/Por, MW cutoff 1K; Type "C" on Pellon). The final sample of 1 to 2 liters of filtered supernatant containing h-EGF was further concentrated by ultrafiltration through an Amicon membrane (YM2, MW cutoff 2k) to yield 200-300 ml.

A column of 1700 ml of packed P-10 resin (Bio-Rad) was equilibrated with 2500 ml of 0.1M acetic acid. The 200 to 300 ml of concentrated hEGF containing filtrate were passed through the column at 4°C at a rate of 0.16 liter per hour. Human EGF was recovered in the included volume of the column., The fractions containing hEGF were pooled (400 ml), and the pool was concentrated using an Amicon ultrafiltration membrane, as previously described (YM2, MW cutoff 2K). About 20 to 30 ml of solution, containing about 16

mg/ml of hEGF was obtained. This solution was then filtered through a 0.45 μm Acrodisk.

The material obtained from the previous step was injected into a C4 reverse phase HPLC column and eluted with a gradient of 5% to 80% acetonitrile in 0.05% trifluoroacetic acid, over approximately 55 minutes. The fractions containing the main hEGF peak were evaporated and freeze-dried. The material was stored as a dry powder.

3. Corneal Epithelial Wounds

The epithelial cells were removed completely from the corneas of anesthestized monkeys by n-octanol and scraping with a Bard-Parker #15 scalpel blade as confirmed by fluorescein staining. Monkeys received two drops three times a day of Neodecadron® alone (control eyes) or containing hEGF (0.1 mg/ml). Each day eyes were stained with fluorescein and photographed. The extent of epithelial regeneration was measured quantitatively by planimetry of enlarged photographs using a Hewlett-Packard distizing table model (9874A). Each wound area was measured three times and the mean value was determined. The extent of epithelial regeneration was expressed as the percentage of the original wound area healed. After 4 days of treatment, monkeys were sacrificed by injection of T-61 euthanasia solution and the corneas removed, fixed in 10% neutral buffered formalin, processed through paraffin, sectioned, and stained with hematoxylin and eosine.

4. Corneal Stromal Wounds

A full thickness central corneal incision 5 mm long was made in both eyes of anesthetized female Macaca fascicularis monkeys. Two drops of Neodecadron® containing hEGF at a final concentration of 0.1 mg/ml were applied to the experimental eyes three times daily. Control eyes were similarly treated with Neodecadron® alone. On days 6 and 9 the corneal

lacerations were examined by slit lamp microscopy and photographed. After 9 days of treatment the extent of wound healing was measured by introducing a 25 gauge cannula into the anterior chamber at the corneal/scleral junction and the intraocular pressure was gradually raised using a Tycos hand-held aneroid manometer. The pressure required to initiate leaking and then bursting of the wound was determined and results were analyzed using the student paired T-test. At the end of the experiment, monkeys were sacrificed by intravenous administration of T-61 euthanasia solution, and eyes were enucleated, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained by hemotoxylin and eosin, and then examined for histological evidence of wound healing.

5. Split Thickness Cutaneous Wounds

Four white Vita-Vet® adult miniature pigs (50-60 lbs) were shaved in the thoracic and paravertebral areas. Each pig was anesthetized with ketamine. Seventy-five 10mm x 10mm split thickness wounds were created on each pig with a modified Castro-Viejo dermatome. The wounds were 0.005 inches deep and were placed 1 cm apart. There were 3 groups of wounds on each pig. Group 1 received topical treatment of saline alone. Group 2 received topical lanolin cream (Squibb and Sons, Princeton, NJ). Group 3 received topical lanolin cream with 10 μg/ml of hEGF. Each wound received 1/2 ml of the appropriate treatment per wound every 12 hours. Twenty-four hours after the initial wounding, four wounds from each group on each pig were entirely excised using a standard blade (22 mm wide). The wound, as well as the surrounding non-wounded skin, was removed. The dermatome was set to a depth of 0.007 inches. The excised specimens were incubated overnight in a 1% trypsin broth at 4°C. The epidermis was easily separated from the dermis on the following day. When no defect was visible in the separated epidermis, the

specimen was considered healed. Four wounds from each group were excised each day for 6 days.

The above experiment was repeated with the exception that Group 2 received treatment with Silvadene® alone and Group 2 received treatment with Silvadene® having hEGF at 10µg/ml.

6. Split Thickness Epidermal Wounds

Four adult miniature pigs (50 to 60 lbs) from Vita-Vet Laboratories, Inc., (Marion, IN) were anesthetized with ketamine and shaved over the dorsal thorax. On each pig, 84 10 mm x 10 mm split-thickness skin wounds were created, 0.005 inches thick and 1 cm apart, with a modified dermatome. On each pig, the wounds were divided randomly into three treatment groups of 28 wounds each. Group 1 received no topical treatment. Group 2 received topical lanolin cream, and group 3 received topical lanolin cream containing 10 µg/ml hEGF. Wounds were treated topically with 0.5 ml of the appropriate cream every 12 hours. The identity of the lanolin and the lanolin-containing hEGF was masked until the results were calculated. Identical experiments were performed using 1 percent silver sulfadiazine in Silvadene® cream.

Every 24 hours after the initial wounding, four wounds from each group on each pig were randomly selected and entirely excised, including 5 mm of surrounding non-wounded skin using a standard 20 mm blade set at a depth of 0.007 inches. The excised specimens were incubated for 24 hours in a 0-.25 percent trypsin broth at 4°C. The epidermis and dermis were readily separated following incubation. The epidermal wound was considered healed when no defect remained in the separated epidermis, or it was considered to be not healed if any defect was present. Results were compared for statistical significance using chi square analysis.

7. Partial Thickness Burns

Four Yorkshire piglets weighing 14 to 20 lbs were obtained locally and were anesthetized with ketamine, shaved over the dorsal thorax and the residual hair removed with dipilatory cream. A 3 cm x 5 cm brass template weighing 430 g was heated to 70°C in water and placed in contact with the dorsal skin of each pig for 10 seconds, and the resultant blister was removed. Histological evaluation of biopsy specimens confirmed that partial-thickness burns had been produced. Six identical wounds were created on the back of each pig (total burned area 90 cm^2 or 6 percent of body surface area). On each pig, two wounds were treated topically with 1 percent silver sulfadiazine in a water-miscible base plus hEGF (10 µg/ml), two wounds were treated with the cream base alone, and two wounds were not treated. The identity of the creams was masked until the data were analyzed. Wounds were treated every 12 hours (0.5 ml cream/cm^2 burn) for 1 week. At the end of 7 days, the fibrinous coagulum was removed and photographs were taken of each wound. The percentage of the original burn area of each wound that had epithelialized was determined by computerized planimetry of enlarged photographs. Results were compared for statistical significance using one-way analysis of variance. Random biopsies were obtained for histological examination from different areas of the wounds to confirm the presence of regenerated epidermis.

The response of the epidermal regeneration with varying concentrations of hEGF was evaluated using similar techniques. Two Yorkshire piglets were anesthetized and 10 identical split-thickness burns were created as before using a 3 cm x 3 cm brass template weight 147 g. The ten wounds were assigned to five groups with two wounds per group. Each group was treated twice daily with either cream base along, hEGF

in cream base (10 µg/ml, 1 µg/ml, or 0.1 µg/ml), or untreated. After 7 days of treatment, the fibrinous coagulum was removed from each wound, the burns were then photographed and quantitative planimetry performed to measure the percent of peidermal regeneration. Results were analyzed for statistical significance using one-way analysis of variance and Tukey's HSD test. In all the partial-thickness burn experiments, each burn was considered an independent measurement for statistical comparison.

Results

1. Corneal Epithelial Regeneration

Quantitative planimetry of the fluorescein staining region of the corneas indicated during the first two days after removal of the epithelium, hEGF-Neodecadron® significantly ($p<0.05$, Chi square analysis) increased the percentage of corneal surface that had re-epithelialized. During the next two days of treatment (days 3 and 4) the eyes treated with Neodecadron® containing hEGF all healed completely as did 2 of the 4 control corneas treated with Neodecadron® alone. The rates of healing in control corneas treated with Neodecadron® alone were linear in all four control corneas until approximately 80 to 90 percent of the initial wound was healed, then the rate of healing decreased. In contrast, the hEGF-Neodecadron® treated corneas consistently displayed curvilinear healing rates with a rapid initial rate of healing which decreased as the wound neared complete healing.

Histological examination of the regenerated epithelium of Neodecadron®-treated corneas revealed 3 to 5 cell layers with tall cuboidal basal cells with pale-staining cytoplasm, and large irregular nuclei characteristic of proliferating basal epithelial cells in middle to end stages of wound healing. In contrast, hEGF-Neodecadron® treated corneas had 4 to 6 cell

layers with cuboidal, basal cells with dark staining cytoplasm and homogenous nuclei which are typical of a normal epithelium.

2. Corneal Stromal Wound Healing

After nine days of treatment, the pressure which caused leaking in the anterior chamber and then bursting of the corneal wounds was measured. Treatment with hEGF-Neodecadron® significantly increased both the leaking and bursting pressures compared to corneas treated with Neodecadron® alone. Further, treatment with hEGF-Neodecadron® increased wound strength relative to Neodecadron® alone both when the epithelium was intact and when the epithelium was removed at the start of the experiment ($p<0.001$). Complete removal of the corneal epithelium before the laceration was made caused significantly less healing both in hEGF-treated corneas ($p<0.01$) and in the control corneas ($p<0.001$) compared to wounds made in corneas with intact epithelium. This suggests that the presence of epithelium aids the process of stromal wound healing.

Histological examination of the corneal wounds revealed that a plug of epithelial cells extended the full length of the cut in the Neodecadron® treated eye. In contrast, a plug of epithelial cells extended only about one-third of the length of the cut in the hEGF-Neodecadron® treated eye. In the remaining portion of the wound, the edges were no longer clearly discernible, but had merged and strands of fibrous material (presumably collagen) spanned the tract of the wound. Examination of the wounds by slit biomicroscopy indicated that the wounds in the hEGF-Neodecadron® treated eyes were narrow and appeared healed, while the wounds treated with Neodecadron® alone appeared wide and unhealed.

3. Split Thickness Cutaneous Wound Healing

Fifty percent (8) of the wounds treated with hEGF-lanolin (N=16) were healed at day 2. The lanolin alone and saline-treated control groups required greater than 4 days before 50% of the excised wounds were healed ($p<0.001$). All wounds (N=16) excised on day 6 were healed. The results were as follows.

	Percentage of Wounds Healed					
Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Saline controls	0%	0%	17%	33%	83%	100%
Lanolin alone	0%	0%	0%	40%	80%	100%
hEGF-Lanolin	0%	50%	83%	100%	100%	100%

Treatment with hEGF-Silvadene® resulted in 62% healing on the second day and 100% healing by the fifth day. In contrast, treatment with saline or Silvadene® alone resulted in no healing until the fourth day and complete healing only after seven days. The results were as follows:

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Saline controls	0%	0%	0%	36%	68%	80%	100%
Silvadene® alone	0%	0%	0%	36%	44%	70%	100%
hEGF-Silvadene®	0%	62%	70%	75%	100%	100%	100%

4. Full Thickness Cutaneous Wound Healing

Twenty male Spraque Dawley rats weighing 350g were included in this study. On the dorsum of each animal, a 3cm interscapular skin incision through the panniculus carnosus was created in order to simulate a primary surgical wound. Ten of the animals were randomly assigned to group A, while the remaining ten animals were assigned to group B. Each animal of group A received 1 cc of "ointment A" and the animals of group B received a like amount of "ointment B." The ointment was applied in the base of the wound prior to

closure of the wound with metal staples. The study was double-blinded, where "ointment A" consisted of 10µg/ml of hEGF in K-Y jelly and "ointment B" consisted of K-Y jelly alone.

Each animal was housed separately, and ten days post-incision the animals were sacrificed in a CO_2 chamber. The wounds were entirely excised with the panniculus carnosus in a 3cm x 3cm rectangle. Those animals in group A had no evidence of the incision when viewed dermal-side up. The animals in group B, however, had a visible defect remaining where the incision was placed when viewed dermal-side up. Burst strength measurements and hydroxyproline assays were all higher in the animals in group A than those in group B. These data indicate that hEGF enhances collagen synthesis as well as epithelialization, and, therefore, is potentially applicable in the treatment of many different types of surgical incisions.

5. Split Thickness Epidermal Wounds

Fifty percent of the hEGF-lanolin-treated epidermal wounds (n=16) were healed at 2 days; whereas, the untreated and lanolin-treated control groups required greater than 4 days for 50 percent of the wounds to heal. All wounds in each group (n=16) were healed by day seven. Similarly, 60 percent of wounds treated with hEGF in 1 percent silver sulfadiazine in a water-miscible base were healed at 2 days; whereas, for 60 percent of the untreated wounds and 60 percent of controls treated with the cream base alone, more than 4 days were required for the wounds to heal. All wounds were healed at the end of 1 week. In both cases the wounds treated with hEGF significantly ($P < 0.01$) accelerated epidermal regeneration compared with untreated wounds or wounds treated with the cream base alone. Of the wounds that were healed, both control wounds and hEGF-treated wounds were similar in histological appearance after 7 days with no evidence

of hyperplastic or metaplastic transformation in the hEGF-treated wounds. All three groups exhibited a 10 to 12-cell layer of stratified squamous epithelium characteristic of normal pig skin.

6. Partial Thickness Burns

After 7 days, virtually complete epidermal regeneration had occurred on the partial-thickness burns treated with hEGF in 1 percent silver sulfadiazine in a water-miscible base, while substantial areas of the untreated burns or the burns treated with the base cream alone were not healed. Results of quantitatively planimetry measurements revealed a statistically significant ($P < 0.01$) increase in the percentage of epithelialization as a result of treatment with hEGF. The results were as follows:

Treatment	Percentage of Burn Area healed*
hEGF	93 ± 6
Control	36 ± 18
Untreated	17 ± 11

*($P < 0.01$) using one-way analysis of variance and Tukey's HSD test. Quantitative planimetry measurements were performed on photographs of partial-thickness burns that were treated for 7 days with hEGF in 1 percent silver sulfadiazine in a water-miscible base (10 µg/ml), 1 percent silver sulfadiazine in a water-miscible base alone (control) or untreated. Values are mean ± SD for 8 wounds.

Biopsy specimens taken 7 days after burn injury confirmed complete epidermal regeneration in those areas judged to be healed in the photographs. The regenerated epidermis of hEGF-treated burns displayed stratification, prominent nucleoli with cells, limited intercellular edema, and a moderate dermal inflammatory response that is typical of proliferating epidermis. In contrast, very little

epidermal regeneration was observed in the burns treated with 1 percent silver sulfadiazine in a water-miscible base or the untreated burns that exhibited substantial dermal inflammatory response. Biopsy specimens taken 35 days after burn injury revealed normal epidermal and dermal architecture in all groups with no evidence of metaplasia in burns treated with hEGF.

All concentrations of hEGF as well as the 1 percent silver sulfadiazine in a water-miscible base alone, significantly ($P < 0.05$) increased the percentage of healed burn surface are compared with untreated controls. However, only burns treated with hEGF at 10 µg/ml were significantly ($P < 0.05$) more healed than the burns treated with the cream base alone. Histological evaluation of representative biopsy specimens confirmed the complete epidermal regeneration in areas judged to be healed on photographs of the burns.

According to the present invention, a wound treatment composition is provided which promotes the rapid healing of epithelial wounds, including both cutaneous wounds and corneal wounds. The compositions include a polypeptide product having an amino acid sequence based on the sequence of natural human epidermal growth factor in an amount effective to promote such healing. The compositions are found to enhance the regeneration of both the epithelial layer and underlying stromal layer resulting from deep wounds to the cornea.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Plasmid pYαEGF-23) was deposited at the American Type Culture Collection on August 12, 1983 and granted accession no. 40079.

WHAT IS CLAIMED IS:

1. A method for treating epithelial and stromal wounds to promote healing, said method comprising applying human epidermal growth factor (hEGF) to the wound, where said hEGF is produced by expressing an hEGF gene coding at least substantially for the amino acid sequence of naturally-occurring human epidermal growth factor in a microorganism.

2. A method as in claim 1, wherein the hEGF is produced in yeast.

3. A method as in claim 2, wherein the yeast is transformed with a DNA construct carrying a synthetic hEGF gene under the transcriptional and translational control of regulatory sequences including secretory leader and processing signal sequences recognized by yeast.

4. A method as in claim 1, wherein the hEGF is applied to a corneal epithelial wound.

5. A method as in claim 1, wherein the hEGF is applied to a corneal stromal wound.

6. A method as in claim 1, wherein the hEGF is present in a physiologically acceptable carrier at from 1μg/ml to 10 mg/ml.

7. A method for treating epithelial and stromal wounds to promote healing, said method comprising applying to the wound a polypeptide capable of stimulating the proliferation of epithelial and stromal cells, where said polypeptide is produced by growing in a suitable culture medium, a yeast host which has been transformed by an extrachromosomal

element carrying a gene coding at least substantially for the amino acid sequence of naturally-occurring human epidermal growth factor under the transcriptional and translational control of regulatory sequences including secretory leader and processing signal sequences recognized by yeast, isolating the polypeptide from the culture medium, and purifying the polypeptide.

8. A method as in claim 7, wherein the hEGF gene is synthetic.

9. A method as in claim 8, wherein the hEGF gene is a synthetic gene composed of preferred yeast codons.

10. A method as in claim 7, wherein the polypeptide is applied to a corneal epithelial wound.

11. A method as in claim 7, wherein the polypeptide is applied to a corneal stromal wound.

12. A method as in claim 7, wherein the polypeptide is present in a physiologically acceptable carrier at from 1µg/ml to 10 mg/ml.

13. A composition for the treatment of epithelial and stromal wounds, said composition comprising:

a polypeptide capable of stimulating the proliferation of epithelial cells, where said polypeptide is produced by growing in a suitable culture medium, a yeast host which has been transformed by an extrachromosomal element carrying a gene coding at least substantially for the amino acid sequence of naturally-occurring human epidermal growth factor under the transcriptional and translational control of

regulatory sequences including secretory leader and processing signal sequences recognized by yeast, isolating the polypeptide from the culture medium, and purifying the polypeptide; and

a physiologically acceptable carrier.

14. A composition as in claim 13, wherein the hEGF gene is synthetic.

15. A composition as in claim 14, wherein the hEGF gene is a synthetic gene composed of preferred yeast codons.

16. A composition as in claim 13, wherein the polypeptide is present in the carrier at from 1μg/ml to 10 mg/ml.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/02024

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL [4] A61K 37/36
U.S. CL 424/95

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols	
U.S.	424/95,912 435/68,172.3,317 935/6,8,37	536/27 514/12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y,P	N, Proceedings of the National Academy of Sciences, Volume 81, issued 1984 August (Washington D.C. U.S.A) A.J. Brake et al., 'X-Factor-directed Synthesis and Secretion of Mature Foreign Proteins in Saccharomyces Cerevisiae', see pages 4642-4646.	1-16
Y	N, Cell, Volume 30, issued 1982, October (Cambridge Massachusetts, U.S.A.) J, Kurjan et al., 'Structure of a Yeast Pheromone Gene (MFx): A Putative X-Factor Precursor Contains Four Tandem Copies of Mature x-Factor', see pages 933-943.	1-16
Y	N, Nucleic Acids Research, Volume 10, no. 15, issued 1982 Coxford England J. Smith et al., 'Chemical synthesis and Cloning of a Gene for Human -Urogastrone' see pages 4467-4482.	1-16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
03 January 1986	09 JAN 1986
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Thomas D. Wamsley for Elizabeth C. Weimar

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	N, Science, Volume 219, issued 1983, February (Washington, D.C. U.S.A), R.A. Hitzeman et al., 'Secretion of Human Interferons by Yeast', see pages 620-624.	1-16
Y	N, Annual Review of Biochemistry, Volume 48, issued 1979, G. Carpenter et al., 'Epidermal Growth Factor', see pages 193-216.	1-16
Y	EP,A, 0,046,039 (G.D. Searle & Co.) published 17 February 1982, see page 2, lines 15-17.	1-16
Y	N, Methods in Enzymology, Volume 68, issued 1979 (New York, NY U.S.A.) R.V. Rothstein et al., 'Synthetic Adaptors for Cloning DNA', see pages 98-109.	1-16
Y	EP,A, 0116201 (Chiron Corporation) published 22 August 1984, see pages 13 and 14.	1-16
Y	N, Chemical Abstracts, Volume 79, issued 1973 September 3 (Colombus Ohio, U.S.A) G. Lucchetti, 'Cicatrizing activity of urogastrone', see page 43, column 1, the abstract no. 49437t, Farmaco Ed. Prat. 1973,28(6), 339-344 (Ital).	1-3,6-9 12-16
Y,P	N, Chemical Abstracts, Volume 102, No. 17, issued 1985 April 29 (Colombus Ohio, U.S.A) J. R. Brigthwell et al. 'Biosynthetic human EGF accelerates healing of neodecadron-treated primate corneas', see page 119, column 1, abstract no. 143835m, Invest. Opthalmol. Visual Sci. 1985 26(1), 105-110 (Eng.).	1-16

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y,P	N, Chemical Abstracts, Volume 102, no. 7, issued 1985 February 18, (Colombus Ohio, U.S.A) T. Kawaba et al. 'Effect of human EGF and plasmia fibronectin on corneal epithelial regeneration', see page 124, column 2, abstract No. 5670h, Nippon Ganka Gakkai Zasshi 1984 88(a), 1237-1249 (Japan).	1-16
Y,P	N, Chemical Abstracts, Volume 102, no. 19, issued 1985 May 13 (Colombus Ohio, U.S.A) P.G. Woost et al. 'Effect of Growth Factors with dexamethasone on healing of rabbit colneal stromal incisions', see page 127, column 1, abstract no. 16137q, Exp. Eye Res. 1985, 40(1), 47-60 (Eng.)	1-3,5-9 11-16
Y	N, Science, Volume 189, issued 1975 (Washington D.C. U.S.A.) R.H. Starkey et al., 'Epidermal Growth Factor: identification of a new hormore in human urine', see pages 800-802.	1-16
Y	N, Graefes Archiv. Ophthalmologie, Volume 210, issued 1979, S. Daniele et al., 'The effect of the Epidermal Growth Factor (EGF) on the Corneal Epithelium in Humans', see pages 159-165.	1-16